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Genetic Basis of Coaggregation Receptor Polysaccharide Biosynthesis in *Streptococcus sanguinis* and Related Species

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SUMMARY

Interbacterial adhesion between streptococci and actinomyces promotes early dental plaque biofilm development. Recognition of coaggregation receptor polysaccharides (RPS) on strains of *S. sanguinis*, *S. gordonii* and *S. oralis* by *Actinomyces* spp. type 2 fimbriae is the principal mechanism of these interactions. Previous studies of genetic loci for synthesis of RPS (*rps*) and RPS precursors (*rml*, *galE1* and *galE2*) in *S. gordonii* 38 and *S. oralis* 34 revealed differences between these strains. To determine whether these differences are strain- or species-specific, we identified and compared loci for polysaccharide biosynthesis in additional strains of these species and in several strains of the previously unstudied species, *S. sanguinis*. Genes for synthesis of RPS precursors distinguished the *rps* loci of different streptococci. Thus, *rml* genes for synthesis of UDP-L-Rha were in *rps* loci of *S. oralis* strains but at other loci in *S. gordonii* and *S. sanguinis*. Genes for two distinct galactose epimerases were also distributed differently. Thus, *galE1* for epimerization of UDP-Glc and UDP-Gal was in galactose operons of *S. gordonii* and *S. sanguinis* strains but surprisingly, this gene was not present in *S. oralis*. Moreover, *galE2* for epimerization of both UDP-Glc and UDP-Gal and UDP-GlcNAc and UDP-GalNAc was at a different locus in each species, including *rps* operons of *S. sanguinis*. The findings provide insight into cell surface properties that distinguish different RPS-producing streptococci and open an approach for identifying these bacteria based on the arrangement of genes for synthesis of polysaccharide precursors.

Keywords

biofilm; coaggregation; galactose epimerase; interbacterial adhesion; receptor polysaccharide; *rps* locus; *Streptococcus sanguinis*

INTRODUCTION

Early evidence for the important role of interbacterial adhesion in dental plaque biofilm formation came from the extensive network of coaggregations observed between strains of streptococci and actinomyces isolated from tooth surfaces (Cisar *et al.*, 1979). The principle mechanism of these interactions is binding of specific streptococcal cell wall polysaccharides, referred to as coaggregation receptor polysaccharides (RPS) by adhesins of other bacteria such as *Actinomyces* spp. type 2 fimbriae. Seven structural types of RPS have been identified from over 25 coaggregating strains of *S. sanguinis*, *S. gordonii*, *S. oralis*

(Cisar *et al.*, 1997) and *S. cristatus* (Yang *et al.*, 2011). Assembly of these polysaccharides depends on synthesis of specific hexa or heptasaccharide repeating units from nucleotide-linked sugars and subsequent polymerization of these units to form long polysaccharide molecules. RPS repeating units contain host-like disaccharide motifs, either GalNAc β 1-3Gal (Gn) or Gal β 1-3GalNAc (G), that function as recognition sites for interbacterial adhesion (Cisar *et al.*, 1995); other features determine RPS serotype. The seven known types of RPS are designated 1Gn, 2Gn, 2G, 3Gn, 3G, 4Gn and 5Gn to indicate their serotype (i.e. 1, 2, 3, 4 or 5) and receptor type (i.e. Gn or G). All RPS serotypes contain Galp, Galf and GalNAc. In addition, Glc and L-Rha occur in RPS serotypes 1, 2 and 3 and ribitol occurs in serotypes 4 and 5.

Molecular studies of the chromosomal locus (*rps*) for RPS biosynthesis in *S. gordonii* 38 (Xu *et al.*, 2003, Yoshida *et al.*, 2005) and in *S. oralis* 34 (Yoshida *et al.*, 2006) revealed closely related genes for the transferases and polymerases that determine RPS structure along with *glf* for galactose furanose mutase, the enzyme that converts UDP-Galp to UDP-Galf (Fig. 1). The location however, of additional genes for synthesis of RPS precursors differed between strains. Thus, *rml* genes for synthesis of TDP-L-Rha were in the *rps* locus of *S. oralis* 34 but at other loci in *S. gordonii* 38. Further characterization of the latter strain revealed genes for two galactose epimerases; *galE1* was downstream of *galK* and *galT* in the galactose operon and *galE2* was downstream of *rmlA*, *rmlC* and *rmlB* in an operon for synthesis of polysaccharide precursors. Importantly, both galactose epimerases catalyzed interconversion of UDP-Glc and UDP-Gal for utilization of galactose via the Leloir pathway (Frey, 1996), but only GalE2 converted UDP-GlcNAc to UDP-GalNAc for RPS biosynthesis (Xu *et al.*, 2003). The occurrence of *galE1* and *galE2* in *S. oralis* 34 was not determined.

We have now compared genetic loci for RPS biosynthesis in additional strains of *S. gordonii* and *S. oralis* and have identified the corresponding loci in several strains of *S. sanguinis*. The results suggest species-specific differences in genetic loci for carbohydrate metabolism and cell surface polysaccharide biosynthesis between the three major species of RPS-producing mitis group streptococci.

METHODS

Streptococcal strains and culture conditions

Table S1 lists all strains of *S. gordonii*, *S. oralis* and *S. sanguinis* considered in the present study. To the best of our knowledge, these strains represent different clones with the possible exception of *S. gordonii* SK120 and SK121, which are colonial variants (Kilian *et al.*, 1989). Whole or partial genome sequences are available for many of these at <http://www.ncbi.nlm.nih.gov/> and <http://www.homd.org/>. Strains from our collection (Hsu *et al.*, 1994) were grown at 37°C in Todd-Hewitt Broth (THB) (Difco Laboratories).

DNA sequencing

Overlapping PCR products were amplified from streptococcal genomic DNA templates using primers designed from gene sequences of *S. gordonii* 38 (Xu *et al.*, 2003) or *S. oralis* 34 (Yoshida *et al.*, 2006) or genomes of *S. sanguinis* SK36, *S. sanguinis* SK355 or *S. oralis* Uo5. Reaction products were purified using QIAquick spin columns (Qiagen) and sequenced at either the NIDCR Sequencing Core Facility or Sequetech (Mountain View, CA, USA). Sequences were assembled and annotated using Vector NTI software (Invitrogen) and compared by BLAST (National Center for Biotechnology).

Comparative PCR studies

PCR with genomic DNA templates from strains of *S. sanguinis* or *S. gordonii* was performed using Platinum PCR SuperMix (Invitrogen) and primers (Table S2) for adjacent genes of *S. sanguinis* SK45 (*nrdG-wzg*, *wefE-galE2-orfP*, *gufA-rmlA* or *orfX-rmlD*) or *S. gordonii* 38 (*nrdG-wzg*, *gufA-rmlA*, *rmlB-galE2-orf9* or *orf15-rmlD-rgpA*). Reaction products were separated by electrophoresis in 0.8% agarose and stained with ethidium bromide.

Plasmid-based genetic complementation

Procedures used for PCR amplification and cloning of *S. sanguinis* SK45 *galE2* into *Escherichia coli*-streptococcus shuttle vector pJY, and transformation of the resulting plasmid (pJY-45) into *S. gordonii* XC8 have been described (Yang *et al.*, 2009) as have those for dot immunoblotting (Yang *et al.*, 2010). Briefly, nitrocellulose membranes were spotted with serial 3-fold dilutions of equivalent *S. gordonii* 38, *S. gordonii* XC8 and *S. gordonii* XC8(pJY-45) suspensions, incubated with affinity-purified rabbit anti-RPS2Gn IgG followed by peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and developed with a Metal Enhanced DAB substrate Kit (Thermo scientific).

RESULTS

Arrangement *rml* genes, *galE1* and *galE2* in *S. gordonii* and *S. oralis*

To determine whether the arrangement of genes for RPS biosynthesis in *S. gordonii* 38 (Fig. 1) is characteristic of this species, we performed comparative PCR studies with primers for adjacent genes of strain 38 and genomic DNA templates from this strain and RPS3G-producing *S. gordonii* strains SK120 and SK121. The PCR products from these strains were indistinguishable by gel electrophoresis (results not shown), thereby indicating that as in strain 38 (Fig. 1), the *rps* locus of both SK120 and SK121 were downstream of *nrdG* and that *rmlA*, *rmlC*, *rmlB* and *galE1* were between *gufA* and *orf9* with *rmlD* between *orf15* and *rgpA*. A *galE1*-containing galactose operon, comparable to that of strain 38 (Xu *et al.*, 2003), was also identified in each RPS3G-producing strain of *S. gordonii*.

We also searched the sequenced genome of *S. gordonii* Challis for genes comparable to those present in strain 38. Although strain Challis does not produce a G- or Gn type of RPS, it does have an uncharacterized operon (bp 2,093,657 to 2,082,323) for *wzy*-dependent polysaccharide biosynthesis immediately downstream of *nrdG*. In addition, *rmlA*, *rmlC*, *rmlB*, and *galE2* are at a separate locus (bp 1,058,910 to 1,062,652) with *rmlD* further downstream (bp 1,070,475 to 1,071,326). A galactose operon containing *galK*, *galT* and *galE1* (bp 967,706 to 971,632) was also identified. Thus, genetic loci for the synthesis of TDP-L-Rha, UDP-Gal and UDP-GalNAc were comparable between *S. gordonii* Challis and three RPS-producing strains of this species.

Examination of nine available whole or partial *S. oralis* genomes revealed a typical *rps* operon between *dexB* and *aliA* in each of six strains, including an *rps2Gn* locus (Xu *et al.*, 2003) in strain SK100, an *rps2G* locus (Yoshida *et al.*, 2005) in strain SK313, an *rps3G* locus (Yoshida *et al.*, 2008) in strains ATCC35037 (SK23) and SK10 and, an *rps4Gn* locus (Yang *et al.*, 2009) in strains SK610 and Uo5. Different operons for synthesis of other polysaccharides were present in the remaining three strains (SK255, SK304 and SK1074). All *S. oralis* strains had a full complement of *rml* genes except SK255 and RPS4Gn-producing SK610 and Uo5. These genes, when present, were always at the 3'-end of polysaccharide gene clusters and arranged as in *S. oralis* 34 (Fig. 1), with *rmlD* transcribed from the opposite strand. We also searched each genome for homologues of *S. gordonii* GalK, GalT, GalE1, and GalE2 to identify genes involved in UDP-Gal and UDP-GalNAc

biosynthesis. All *S. oralis* genomes contained closely linked *galK* and *galT* but surprisingly, none contained *galE1*. This species did however contain *galE2*. Eight of nine *S. oralis* genomes had a single copy of this gene at a locus like the one in *S. oralis* 34 (Fig. 1). The remaining strain (SK304) contained two copies of *galE2*, one at a locus like that in other *S. oralis* strains and the other at a different locus (ctg120008662248, nucleotides 236395 to 237405) like one present in certain strains of *S. mitis* and *S. pneumoniae*.

Genetic basis of RPS biosynthesis in *S. sanguinis*

We then turned our attention to *S. sanguinis*, a previously unstudied species. The *rps1Gn* locus identified from *S. sanguinis* SK45 and that of *S. oralis* 34 were syntenous from *wzg* for a transcriptional regulator to *wefE* for the last glycosyltransferase (Fig. 1). The gene downstream of *wefE* was however, *rmlA* in *S. oralis* 34 versus a *galE* homologue in *S. sanguinis* SK45. The encoded *S. sanguinis* protein was 58% identical to GalE2 of *S. gordonii* 38 (and *S. oralis* 34) and 43% identical to GalE1 of *S. gordonii* 38. Identification of the *S. sanguinis* SK45 gene as *galE2* was confirmed by its ability, when expressed from pJY-45, to restore RPS production by a *galE2* mutant (strain XC8) of *S. gordonii* 38 (Fig. 2). Further sequencing of *S. sanguinis* SK45 revealed *rmlA*, *rmlC* and *rmlB* at one locus and *rmlD* at another (Fig. 1) as well as a galactose operon (not shown in Fig. 1) containing *galK*, *galT* and *galE1* (GalE1 of *S. sanguinis* SK45 and *S. gordonii* 38 are 96% identical). Thus, the only significant difference noted between *S. sanguinis* SK45 and *S. gordonii* involved the location of *galE2*.

To determine whether findings with *S. sanguinis* SK45 were typical of this species, we performed comparative PCR studies with primers for adjacent genes of strain SK45 and genomic DNA templates from this and six other RPS1Gn-producing *S. sanguinis* strains (Table S1, 16 to 21). The PCR products observed from all strains were indistinguishable by gel electrophoresis (results not shown). Related genes were also identified in 21 whole or partial *S. sanguinis* genomes. Twenty of these had *galE1*-containing galactose operons and *rml* loci like those of strain SK45. The only exception was *S. sanguinis* ATCC49296. This strain, which lacked *galE1* and *rml* genes but contained an *rps4Gn* locus and *galE2*, was not like any other strain of *S. sanguinis*. Instead, it resembled *S. oralis* SK610 and Uo5 (Table S1), which supports earlier suggestions (Narikawa *et al.*, 1995, Zahner *et al.*, 2011) that strain ATCC 49296 has been misclassified as *S. sanguinis*. Six typical *S. sanguinis* strains (Table S1, 21 to 26) had *rps1Gn* loci that included *galE2*, as in strain SK45 (Fig. 1). Additional copies of *galE2* were not identified in genomes of these strains. The remaining 14 strains (Table S1, 27 to 40) lacked *rps1Gn* loci but instead had other operons for polysaccharide biosynthesis downstream of *nrdG*. The nucleotide sequences of these operons, although similar between strains (~90% identical), were distinguished by the presence of *galE2*, which was the last gene in the operons of eight strains (Table S1, 27 to 34) but was not present in the other six strains (Table S1, 35 to 40). Thus, when present, the location of *galE2* at the 3'-end of a polysaccharide gene cluster distinguished *S. sanguinis* from *S. gordonii* and *S. oralis*.

DISCUSSION

Differences in the genetic loci for synthesis of polysaccharide precursors between RPS-producing strains of *S. gordonii*, *S. sanguinis* and *S. oralis* provide insight into cell-surface properties that distinguish these streptococci. Synthesis of complex polysaccharides depends on an array of genes, some that contribute only to polymer synthesis and other so-called housekeeping genes that have broader metabolic roles (Bentley *et al.*, 2006). The former genes typically occur in large operons, such as those for RPS biosynthesis, with the latter at other loci. Thus, the location of *rml* genes in different streptococci (Fig. 1) suggests that the

requirement for TDP-L-Rha is limited to RPS biosynthesis in *S. oralis*, but extends to other pathways in *S. sanguinis* and *S. gordonii*. Support for this interpretation comes from production of an L-Rha-containing polysaccharide of the type described from *S. mutans* (Yamashita *et al.*, 1998) by *S. sanguinis* and *S. gordonii* (Cisar *et al.*, 1997). Genes for synthesis of these polysaccharides, which include *rgpA*, are downstream of *rmlD* in *S. sanguinis* and *S. gordonii* (Fig. 1) but not present in genomes of *S. oralis*.

In previous studies of *S. gordonii* 38 (Xu *et al.*, 2003), we showed that *galE2* is essential for RPS biosynthesis and attributed this to the unique ability of GalE2 to supply UDP-GalNAc. In the present study, we identified a *galE* homologue in the *rps1Gn* operon of *S. sanguinis* SK45 based on its ability to restore RPS production in a *galE2* mutant of strain 38 (Fig. 2). Similar *galE* homologues occur in *rps1Gn* operons as well as other operons for polysaccharide biosynthesis in many but not all sequenced *S. sanguinis* strains (Table S1). With the exception of atypical ATCC49296, these strains also harbor galactose operons that contain homologues of *S. gordonii* 38 *galE1*. The only other homologous genes in *S. sanguinis* are *rmlB* and *rmlC*, which have well established roles in L-Rha biosynthesis. From this, we suspect that the supply of UDP-GalNAc for polysaccharide biosynthesis in this species depends solely on expression of *galE2* from *rps1Gn* operons (and other polysaccharide gene clusters). The location of *galE2* in *rps1Gn* operons of *S. sanguinis* (Fig. 1) but outside similar operons in *S. oralis* can be explained by the presence or absence of *galE1* in these species, respectively. Thus, the presence of *galE1* in *S. sanguinis* may limit the role of *galE2* to RPS biosynthesis while the absence of *galE1* in *S. oralis* may make *galE2* essential for RPS biosynthesis and galactose metabolism. The broader role of *galE2* in *S. oralis* is consistent with the ability of this gene to fulfill both roles in *galE1* mutants of *S. gordonii* (Xu *et al.*, 2003).

Molecular phylogenetic analysis of encoded protein sequences offered additional support for the present identification of streptococcal *galE* homologues as *galE1* or *galE2*. Thus, the deep separation of GalE1 and GalE2 sequences (Fig. 3) is consistent with previous phylogenetic and structural studies of the UDP-hexose 4-epimerase family (Ishiyama *et al.*, 2004), which suggest that differences in substrate preference appeared early in the evolution of these enzymes. Further evidence for this difference was obtained by comparing these sequences with those of *E. coli* K12 GalE (Thoden *et al.*, 2002), *Trypanosoma brucei* GalE (Roper *et al.*, 2005) and human GalE (Thoden *et al.*, 2002). The human protein has substrate specificity like *S. gordonii* 38 GalE2 whereas the other two have substrate specificity like *S. gordonii* 38 GalE1. The different substrate preference of *E. coli* K12 and human GalE depends to a significant extent on specific amino acid residues that function as gatekeepers for entry of acetylated substrates into the catalytic sites of these enzymes (Thoden *et al.*, 2001). Thus, the bulky side chain of Tyr299 in *E. coli* GalE allows entry of smaller unacetylated substrates but blocks entry of larger acetylated substrates whereas the smaller side chain of Cys309 in human GalE allows entry of both unacetylated and acetylated substrates. In *T. brucei* GalE, which is more closely related to streptococcal GalE1 than *E. coli* K12 GalE (Ishiyama *et al.*, 2004), the equivalent residue is Leu342 (Shaw *et al.*, 2003). Significantly, Leu also occurs at the corresponding position in all streptococcal GalE1 sequences. In contrast, Cys as in human GalE, is present in all streptococcal GalE2 sequences except those of *S. gordonii* GalE2, which have Ser, a residue of similar size. These findings strongly support identification of both *galE1* and *galE2* based on their location in the streptococcal chromosome, and thereby strengthen the conclusion that the presence of *galE1* in *S. sanguinis* and *S. gordonii* distinguishes these species from *S. oralis*.

RPS-bearing streptococci are prominent early colonizers of the tooth surface where they appear in micro-colonies along with other cell types including type 2 fimbriated *Actinomyces* spp. (Palmer *et al.*, 2003) and non-RPS producing strains of *S. gordonii* that

bind Gn-types of RPS (Chalmers *et al.*, 2008). While *S. oralis* is the major RPS-producing species (Hsu *et al.*, 1994), strains identified as *S. sanguinis*, *S. gordonii*, *S. cristatus* (Yang *et al.*, 2011), and *S. australis* (J. Cisar, unpubl. obs.) also produce these polysaccharides. Three RPS-producing strains of *S. mitis* (i.e. strains J22, ATCC15914 and H127) were also reported previously (Cisar *et al.*, 1997). Subsequent taxonomic studies have however identified strain J22 as *S. oralis* (Kilian *et al.*, 2008) and strains ATCC15914 and H127 as *S. oralis* and *S. australis*, respectively, (M. Kilian, personal communication). Production of Gn- or G-types of RPS by strains of *S. oralis* but not *S. mitis* is consistent with the presence of identifiable *rps* operons in six of the nine available *S. oralis* genomes (Table S1, 6 to 14) but not in any of 12 *S. mitis* genomes (J. Cisar unpubl. obs.). Related operons for cell-surface polysaccharide biosynthesis do however occur in many strains of *S. mitis* (Kilian *et al.*, 2008). These operons may well direct synthesis of polysaccharides that represent a novel type of RPS, such as one identified from *S. mitis* SK137 (Bergstrom *et al.*, 2000). Accordingly, evolution of *S. oralis* and *S. mitis* could reflect the association of these closely related streptococci with different members of the plaque biofilm community.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of *rps* locus of *S. sanguinis* SK45 (GenBank HM046485.1) with those of *S. gordonii* 38 (GenBank AY147914.1) and *S. oralis* 34 (GenBank AB181234.2) showing percent identity between encoded regulatory proteins (red), glycosyl transferases (blue), polysaccharide polymerases (white), flippases (black), enzymes for synthesis of nucleotide-linked sugars (green) and flanking genes (gray). Synthesis of TDP-L-Rha and UDP-GalNAc depend on additional loci in *S. gordonii* 38 (GenBank AY147913.1), *S. sanguinis* SK45 (GenBank JX407104.1, JX407105.1) and *S. oralis* 34 (GenBank KC620451). The presence of *wefB* and *wefC* in the *rps2Gn* operon of *S. gordonii* 38 versus *wefH* in the *rps1Gn* operons of *S. sanguinis* SK45 and *S. oralis* 34 is associated with the structural difference between RPS2Gn and RPS1Gn.

Figure 2.

Detection of cell surface RPS on *S. gordonii* 38, *S. gordonii* XC8 and *S. gordonii* XC8(pJY-45) by dot immunoblotting with rabbit anti-RPS2Gn antibody. Expression of *S. sanguinis* SK45 *galE2* from pJY45 restored RPS production in *S. gordonii* XC8, a *galE2* mutant of wild type strain 38.

Figure 3.

Molecular phylogenetic analysis of full length GalE protein sequences as deposited in GenBank (Table S1) from strains of *S. gordonii* (Sg), *S. oralis* (So) and *S. sanguinis* (Ss) showing separation of GalE1 and GalE2 in an unrooted tree constructed by Maximum Likelihood method using MEGA5 (Tamura et al., 2011). Bootstrap values (%) are based on 1500 replications; the scale bar refers to genetic divergence as calculated by the MEGA software. The ability of human GalE and inability of *E. coli* (Ec) GalE to act on acetylated substrates depends in part on C309 in the former protein and Y299 in the latter. C or S occurs at the equivalent position in all streptococcal GalE2 sequences. In contrast, L occurs in all GalE1 sequences and in *T. brucei* (Tb) GalE (L342), which has substrate specificity like that of *E. coli* GalE.